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Serotonin Transporter Promoter Polymorphism Is Associated With Attenuated Prolactin Response to Fenfluramine

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Disturbances in central serotonin (5-HT) function may have a role in impulsive aggression in patients with a wide range of psychiatric diagnoses. The underlying mechanism, however, remains unknown. There are several naturally occurring mutations in the 5-HT signaling pathway that may underlie differences in 5-HT function and responsivity to drugs that affect 5-HT functioning. In the present study, we examined the relationship between polymorphisms in the promoter region of the gene coding for the neuronal 5-HT transporter, fenfluramine-induced prolactin release, and aggressive impulsivity (as measured by Barratt Impulsivity Scale, Buss-Durkee Hostility Inventory, and Brown-Goodwin Aggression Scale scores), in a group of abstinent alcoholic patients and healthy volunteers. We report here that possession of the short variant of the 5-HT transporter promoter polymorphism was associated with a blunting of overall central 5-HT function, as measured by fenfluramine-induced prolactin release. We found no relationship between aggressive, hostile, or impulsive traits and fenfluramine-induced prolactin release or between these traits and polymorphisms in the 5-HT transporter promoter. Thus, we have shown that a 5-HT transporter promoter genotype, which has previously been associated with anxiety-based behaviors, alters an in vivo measure of central 5-HT function (fenfluramine-induced prolactin release), providing an important mechanism for linkage between a gene, physiological function, and behavior. Published 2001 Wiley-Liss, Inc.†

KEY WORDS: 5-HT; HTTLPR; impulsivity; aggression; alcoholism

INTRODUCTION

Over the last 20 years, evidence has accumulated suggesting that disturbances in central serotonin (5-hydroxytryptamine, 5-HT) function may have a role in major depressive disorders, suicide attempts, early-onset alcoholism, binge eating, unprovoked interpersonal violence, and impulsive aggression in patients with a wide range of psychiatric diagnoses. The association of 5-HT with this heterogeneous array of disorders indicates that reduced central 5-HT functioning may be correlated with a general trait of irritable impulsive aggressive behavior, rather than a specific psychopathological category [Coccaro, 1992]. Although such correlations between 5-HT and behavior are well documented, the mechanisms by which decreased 5-HT activity is related to impulsive aggression are currently unknown.

Insights into central 5-HT function can be gained by measuring 5-HT–induced prolactin release. Because 5-HT itself does not cross the blood-brain barrier, central 5-HT activity can be increased indirectly by administration of compounds including 5-HT precursors, 5-HT–releasing agents, selective 5-HT uptake inhibitors, and direct 5-HT agonists. D-fenfluramine has proven to be particularly useful as a challenge drug for measuring net or overall 5-HT activity. Fenfluramine

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increases extracellular 5-HT concentrations by reversing the usual outside-to-inside directionality of the 5-HT transporter in the plasma membrane of serotonergic terminals, thus simultaneously releasing 5-HT and blocking its uptake [Wolfel and Graefe, 1992; Gobbi et al., 1993; Coccaro et al., 1998]. The resultant increased extracellular 5-HT levels stimulate postsynaptic 5-HT receptors in the paraventricular and supraoptic nuclei of the hypothalamus, which in turn stimulate the release of pituitary hormones including adrenocorticotropic hormone and prolactin from the anterior pituitary, and oxytocin and vasopressin from the posterior pituitary [Levy and Van de Kar, 1992]. The available evidence suggests that prolactin secretion requires concurrent stimulation of postsynaptic 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors [Meltzer and Maes, 1995]. Thus, fenfluramine-stimulated prolactin release depends not only on the density of presynaptic 5-HT transporters, but also on the density/activity of at least three 5-HT receptor subtypes, making this response a convenient, integrated measure of central 5-HT system function.

Coccaro [1992] has reviewed a series of studies showing that patients with violent suicidal and/or impulsive aggressive behaviors exhibit decreased fenfluramine-induced prolactin release, suggesting reduced overall 5-HT function in the limbic hypothalamic system of these patients. These findings were further confirmed by the demonstration of impaired fenfluramine-induced prolactin release in convicted murderers with a diagnosis of antisocial personality disorder [O'Keane et al., 1992], impulsive/aggressive patients with posttraumatic stress disorder [Southwick et al., 1999], and men rated as highly aggressive [Netter et al., 1999]. Although this relationship between decreased central 5-HT function and impulsive aggression is typically restricted to patients diagnosed with a personality disorder, a recent report indicates that in a community-derived sample of normal men, heightened aggression and impulsivity were significantly correlated with low prolactin responses to fenfluramine challenge [Manuck et al., 1998].

Recent advances in the molecular biology of 5-HT signaling systems have identified several mutations that may have a role in the link between central 5-HT function and psychopathology. The human serotonin transporter gene has been shown to contain two common polymorphisms: a variable number of tandem repeats in intron 2 and a functional insertion/deletion in the promoter region [Heils et al., 1996]. The promoter polymorphism is biallelic, with the short allele associated with less promoter activity and reduced 5-HT transporter gene activity in an in vitro lymphoblastoid expression system [Heils et al., 1996]. Many recent studies suggest that the possession of the short allele is associated with a variety of disorders, including increased susceptibility to depression and anxiety [Lesch et al., 1996; Ohara et al., 1998; Flory et al., 1999], both early and severe alcohol dependence [Hallikainen et al., 1999], and decreased susceptibility to obsessive-compulsive disorder [Bengel et al., 1999]. Because fenfluramine acts directly at 5-HT transporters to release 5-HT, it is possible that the alterations in the expression of the 5-HT transporter expected from differing 5-HT transporter promoter variants will affect fenfluramine-induced prolactin release and that patients with such alterations in their 5-HT transporter expression will exhibit decreased prolactin response. Thus, fenfluramine-induced prolactin release may provide the physiological link between 5-HT promoter polymorphisms and the behavioral traits with which they have been associated.

In the present study, we examined the relationship of the 5-HT transporter promoter genotype to fenfluramine-induced prolactin release, measures of aggression, impulsivity, and hostility in a sample of male volunteers.

MATERIALS AND METHODS

Subjects

The study was approved by the Human Subjects Committee of the Long Beach Veterans Affairs Healthcare System. All subjects provided informed consent to participate. Twenty-seven male subjects were recruited from volunteers who had participated in an earlier study in which genotyping of the serotonin transporter had been conducted. These subjects had been recruited from a residential treatment unit for alcohol dependence or were nonpatient volunteers. All subjects had been sober for at least 3 weeks at the time of testing.

Baseline assessment began with a psychiatric and medical history followed by the following rating instruments: Barratt Impulsivity Scale (BIS), Buss-Durkee Hostility Inventory (BDHI), Structured Clinical Interview for DSM-III-R (SCID II) Personality Disorder Questionnaire, and Brown-Goodwin Aggression Scale, Modified (BGA). The SCID II questionnaire was followed up with an interview to establish the presence or absence of a personality disorder. Subjects were excluded from the patient group if they had an axis I disorder other than substance abuse, suffered from medical illness, or were receiving psychotropic medication.

Procedures

On the test day, subjects were n.p.o. after midnight. At 08:00, a catheter was inserted in a forearm vein. At 10:00, subjects received fenfluramine (30 mg p.o.). Blood samples were collected every 30 min beginning at 09:30 and ending at 15:00. Subjects were permitted to drink fluids during the test period and remained in the research suite, engaged in quiet activities.

Assays

Fenfluramine-induced Prolactin Release. Plasma levels of fenfluramine and its principal metabolite, norfenfluramine, were measured by gas chromatography with electrochemical detection [Krebs et al., 1984]. The lower limit of detection was 2 ng/mL for fenfluramine and 3 ng/mL for norfenfluramine. Plasma prolactin levels were determined using a commercially available radio immunoassay kit (Diagnostic

TABLE I. Comparison of Data

Serotonin transport promoter gene	Patient group	Nonpatient group	Overall occurrence	
			n	Frequency
Long-long	6	3	9	.33
Short-long	7	4	11	.41
Short-short	1	6	7	.26

System Lab; DSL-10-4500). The primary antibodies used were specific for prolactin as shown by preabsorption and cross-reactivity assays by the manufacturer. The lower limit for detection was 2 ng/ml.

5-HT Genotypes. Polymorphisms in the promoter region of the 5-HT transporter gene were genotyped from genomic DNA prepared from drawn blood samples as previously described [Schuckit et al., 1999; Heinz et al., 2000]. DNA amplification was accomplished with polymerase chain reaction using the two flanking oligonucleotide primers suggested by Heils et al. [1996]. This set of primers amplifies a 484 or 528 bp fragment corresponding to the 5-HT promoter short or long allele. Polymerase chain reaction amplification was carried out in a final volume of 30 μL of a buffer system consisting of 10 mM Tris-HCl (pH 8.3) with 50 mM KCl and 1.5 mM $MgCl_2$, and containing 50 ng genomic DNA from the patient blood samples, 2.5 mmole/L deoxyribonucleotides, 0.1 μg of sense and antisense primers, and 1 U Taq DNA polymerase. The amplification process consisted of 35 cycles of annealing at 61°C for 30 sec, extension at 72°C for 1 min, and denaturation at 95°C for 30 sec.

Statistical Analyses

To determine relationships between 5-HT transporter promoter polymorphisms and fenfluramine-induced prolactin release or behavioral scores, mixed analyses of variance were conducted on the data collected. In the first analysis, genotype was used as a between-subjects factor while timed measurements of circulating prolactin levels were a within-subjects repeated measure. The ANOVA was constructed using 5-HT transporter promoter polymorphism as a between-subjects factor and prolactin response as a within-subjects factor. The 5-HT transporter promoter polymorphisms were categorized as short-short, short-long, and long-long. In a second set of analyses, univariate ANOVAs were performed on genotype and each of the behavioral measures (BGA, BIS, and BDHI).

To determine the relationship between fenfluramine-induced prolactin release and behavioral scores, multiple regression analysis was performed. For all analyses, significance was defined at an alpha level of 0.05.

RESULTS

Subject Characteristics

The 14 subjects recruited from the residential program had a mean age of 40 ± 10.7 years and were diagnosed with alcohol dependence. Ten of these subjects also met criteria for an axis II disorder. The nonpatient group had a mean age of 41.4 ± 11.6 years ($P = 0.75$) and were free of any axis I or II diagnosis. Multivariate analysis of variance showed that the substance abuse subjects scored higher on ratings of aggression (BGA-revised total: 10.9 ± 6.3 vs. 6.1 ± 3.4; $P = 0.02$), impulsivity (BIS: 68.6 ± 17.5 vs. 47.7 ± 21.8; $P = 0.01$), and hostility (BDHI: 47.4 ± 14.7 vs. 25.3 ± 10.8; $P < 0.001$) than did controls.

Neurogenetics

Allele frequencies for the 5-HT transporter polymorphism in our subjects are shown in Table I. Results of the ANOVA performed on fenfluramine-induced prolactin release and 5-HT transporter promoter genotype data revealed that the data violated the assumption of sphericity, thus all degrees of freedom for all subsequent analyses were adjusted by the Greenhouse-Geisser epsilon values calculated. We observed a significant main effect of time on the prolactin response [$F(2.29, 54.88) = 23.80$; $P = 0.000$] and an interaction between genotype and time [$F(4.57, 54.88) = 2.58$; $P = 0.041$]. The power for the interaction was 72.9%. Posthoc analyses using tests for simple effects showed that there was a significant change in prolactin level over time for subjects with the short-long and long-long polymorphisms [$F(2.75, 27.46) = 14.80$, $P = 0.000$, and $F(1.52, 12.16) = 11.34$, $P = 0.003$, respectively; Fig. 1]). The increase in circulating prolactin over time did not reach significance for subjects with the short-short genotype [$F(1.42,8.52) = 3.95$; $P = 0.071$]. The prolactin response for all three genotypes exhibited a significant linear trend [short-short $F(1, 6) = 6.35$, $P = 0.045$; short-long $F(1, 10) = 45.64$, $P = 0.000$; long-long $F(1, 8) = 17.82$, $P = 0.003$]. Analysis of the slopes of the prolactin response showed that subjects with the long-long genotype had greater prolactin increases over time after fenfluramine administration than those with the short-long or short-short polymorphism.

Univariate ANOVA showed no significant correlation between 5-HT transporter promoter genotype and any of the impulsivity/aggression rating scores. Finally, regression analysis showed that there was no significant correlation between fenfluramine-induced prolactin release and any measure of impulsive aggression, with P values ranging from 0.114 for the relationship between prolactin and BDHI total score to 0.323 for prolactin and BIS total score.

DISCUSSION

The frequencies of L- and S-alleles in our subject population agree well with those reported in a series of studies by Gelernter et al. [1997, 1998, 1999]. The L:S

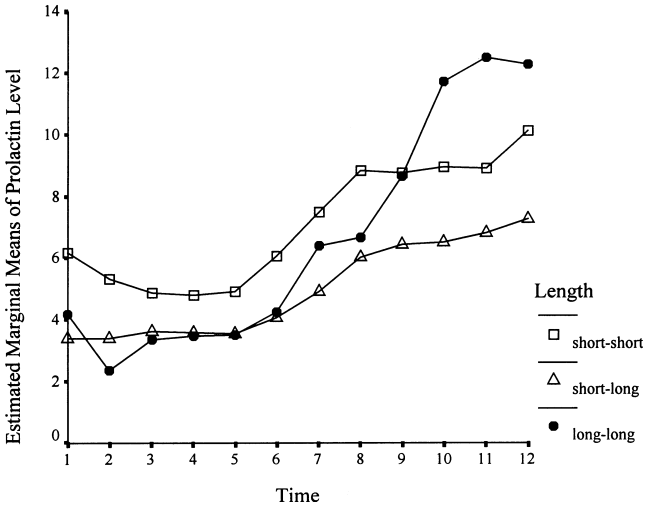

Fig. 1. Fenfluramine-induced prolactin release was blunted in subjects possessing the short allele of the 5-HT transporter promoter. Fenfluramine-induced prolactin release normally increased over time in subjects possessing the long-long allele. This increase was attenuated in subjects with either short-long or short-short polymorphisms.

ratio in the present study is 0.54:0.46. This is close to the 0.59:0.41 ratio reported for 105 European Americans [Gelernter et al., 1997] and almost identical to the 0.53:0.47 ratio later reported for 221 European Americans [Gelernter et al., 1998]. In light of the fact that our subject population was composed of abstinent alcoholics and normal volunteers, it should be noted that neither of these previous studies found a significant difference in allele frequencies between alcoholics [Gelernter et al., 1997] or substance abusers [Gelernter et al., 1998] and controls. The data in the present study also agree reasonably well with the frequencies of specific 5-HT transporter promoter genotypes recently reported by Heinz et al. [2000]. Their study surveyed a small population of alcoholics and control subjects and found overall genotype frequencies of 0.36:0.45:0.18 (LL:LS:SS). This would appear to be similar to the 0.35:0.38:0.27 ratio in the present study, although we have a slightly lower percentage of long-short heterozygotes and an excess of short-short homozygotes. Again, Heinz et al. [2000] reported no significant difference in genotypes between alcoholics and control subjects, which may be a factor in the agreement between their data and that from our abstinent alcoholic/control population.

The major finding of the present study is that fenfluramine-induced prolactin release was blunted in subjects with the short allele of the 5-HT transporter promoter; subjects with the long-long genotype have a more robust prolactin response than those with either long-short or short-short genotypes. These data fit the current model of the promoter polymorphism very well. The short allele has been shown to reduce the transcriptional efficiency of the 5-HT transporter promoter and 5-HT transporter expression in an in vitro cell line [Heils et al., 1996; Lesch et al., 1996] and has been shown to decrease radioligand binding to the 5-HT transporter in the raphe nuclei of patients with short alleles [Heinz et al., 2000]. In a study by William et al. [2001], subjects homozygous for the short allele had CSF 5-HIAA levels 50% lower than subjects possessing at least one long allele. Because fenfluramine acts directly at the 5-HT transporter to release 5-HT, the decreased expression of 5-HT transporters conferred by the short allele would be expected to impair the ability of fenfluramine to release 5-HT and in turn this lower 5-HT release would be expected to diminish prolactin secretion. Heinz et al. [2000] showed that the short allele is associated with lower in vivo 5-HT transporter expression in the raphe nuclei as measured by SPECT. This is consistent with a postmortem study by Little et al. [1998] in which the presence of the short allele was associated with decreased raphe 5-HT transporter mRNA levels. The data in the present study takes one important step farther: we demonstrate that possession of the short allele has significant in vivo physiological consequences, leading to a functional alteration in central 5-HT activity.

Observations of decreased 5-HT function in patients with disinhibited aggression directed at self or others are among the most replicated findings in biological psychiatry [Coccaro, 1992]. Studies employing indexes that primarily reflect presynaptic 5-HT function typically report decreases in these indicators. For example, decreased 5-HIAA levels in cerebrospinal fluid have been found in impulsive, aggressive individuals [Brown and Linnoila, 1990], patients with histories of suicide attempts [Coccaro et al., 1989], short-term abstinent alcoholics [Linnoila et al., 1994], and alcoholic impulsive offenders with either antisocial personality disorder or intermittent explosive disorder [Virkkunen et al., 1994]. Other studies rely on 5-HT indexes that principally reflect postsynaptic function, typically showing increases in these measures. For example, increased radioligand binding to 5-HT_{2A} receptors has been reported in patients with personality disorders and high BDHI assault scores [Coccaro et al., 1997] and for patients with major depression and a history of medically damaging suicidal acts [McBride et al., 1994]. These increases in postsynaptic 5-HT indexes may reflect increases in postsynaptic activity in response to the reductions in presynaptic 5-HT activity noted above. The net effect of such presynaptic decreases and postsynaptic increases on overall central 5-HT functioning is difficult to predict, however. Recent data from our laboratory and others highlight this dilemma. Although McBride et al. [1994] reported an increase in 5-HT_{2A} receptor binding in depressed/suicidal patients, when they measured the ratio of 5-HT–amplified platelet aggregation to 5-HT_{2A} receptor number (an index of the mean responsivity of an individual 5-HT receptor complex), they found that it was lower in suicide attempters versus nonattempters or normal controls. This suggests that while the number of 5-HT_{2A} receptors may be increased in impulsive, aggressive patients, their functionality may be decreased. We have recently found decreased 5-HT–induced Ca^{2+}

release from platelets [Reist et al., 2000], a measure of the function of the 5-HT_{2A} receptor second messenger cascade, in patients with high impulsivity, aggression, and hostility scores in a population of abstinent substance abusers and, using the same subject population as that used in the present study, we observed a significant inverse correlation between impulsivity (as measured by BIS scores) and 5-HT–induced Ca^{2+} release in platelets [Reist et al., 2000].

Fortunately, a more direct assessment of net central 5-HT function can be made via measurements of prolactin release in response to an acute challenge with fenfluramine. Fenfluramine is an indirect 5-HT agonist, releasing endogenous presynaptic stores of 5-HT and blocking the uptake of the released neurotransmitter, thus strongly stimulating postsynaptic 5-HT receptors. The subsequent stimulation of 5-HT_{2A} receptors in the hypothalamic/pituitary system triggers a dose-dependent release of prolactin. As such, measurements of fenfluramine-induced prolactin release integrate both pre- and postsynaptic elements and reflect net 5-HT functioning. Decreased net central 5-HT function has been reported in patients with violent suicidal and/or impulsive aggressive behaviors [Coccaro, 1992]. These findings have been further confirmed by the demonstration of impaired fenfluramine-stimulated prolactin release in convicted murderers with a diagnosis of antisocial personality disorder [O'Keane et al., 1992], in impulsive/aggressive patients with posttraumatic stress disorder [Southwick et al., 1999], and in men rated as highly aggressive [Netter et al., 1999]. Although the subjects used in this study with high impulsivity show decreased postsynaptic 5-HT function, there was no correlation between measures of impulsive aggression in this population and fenfluramine-stimulated prolactin release. This apparent disagreement with the literature reporting decreased fenfluramine-stimulated prolactin release in impulsive, aggressive individuals may be due to the fact that the impulsive, aggressive subjects used in the previous studies have used patients with personality disorders, whereas many of the subjects in the present study were free of underlying personality disorders. Only one previous study has reported an inverse correlation between fenfluramine-induced prolactin release and impulsive aggression in a nonpatient sample [Manuck et al., 1998] and their sample size was more than twice that available for the present study.

Variations in the 5-HT transporter promoter have been controversially associated with anxiety and depressive disorders, but a clear consensus has yet to emerge [Ohara et al., 1998; Deary et al., 1999; Flory et al., 1999; Frisch et al., 1999; Katsuragi et al., 1999; Menza et al., 1999]. In the present study, we observed that polymorphisms in the 5-HT transporter promoter were not correlated with any of the tested impulsivity, hostility, or aggression trait measures. This lack of correlation is not surprising in that none of these behavioral indexes directly address the anxiety/depression typically associated with 5-HT transporter polymorphisms. Our findings are also in agreement with those of Kotler et al. [1999], who reported no association between the serotonin transporter polymorphism and homicidal behavior in schizophrenic patients.

In summary, in the present study we have shown that subjects that possess the short variant of the 5-HT transporter promoter polymorphism exhibit blunted central 5-HT function (as measured by fenfluramine-induced prolactin release). These data provide functional physiological evidence for a link between a serotonergic genotype and the behavioral traits (anxiety and depression) that have been associated with these genotypes.

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